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PHYSIOLOGY/STRUCTURAL BIOLOGY/BIOCHEMISTRY

Cloning and Characteristics of a Gene Encoding NADH Oxidase, a Major Mechanism for Oxygen Metabolism by the Anaerobic Spirochete, *Brachyspira (Serpulina) hyodysenteriae*

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Brachyspira (Serpulina) hyodysenteriae cells consume oxygen during growth under a $1\%O_2:99\%N_2$ atmosphere. A major mechanism of O_2 metabolism by this anaerobic spirochete is the enzyme NADH oxidase (EC 1.6.99.3). In these investigations, the NADH oxidase gene (*nox*) of *B. hyodysenteriae* strain B204 was cloned, expressed in *Escherichia coli*, and sequenced. By direct cloning of a *Hind* III-digested DNA fragment which hybridized with a *nox* DNA probe and by amplification of B204 DNA through the use of inverse PCR techniques, overlapping portions of the *nox* gene were identified and sequenced. The *nox* gene and flanking chromosome regions (1.7 kb total) were then amplified and cloned into plasmid pCRII. Lysates of *E. coli* cells transformed with this recombinant plasmid expressed NADH oxidase activity (1.1 μmol NADH oxidized/min/mg protein) and contained a protein reacting with swine antiserum raised against purified *B. hyodysenteriae* NADH oxidase. The *nox* ORF (1.3 kb) encodes a protein with a predicted molecular mass of 50 158 kDa. The *B. hyodysenteriae* NADH oxidase shares significant (46%) amino acid sequence identity and common functional domains with the NADH oxidases of *Enterococcus faecalis* and *Streptococcus mutans*, suggesting a common evolutionary origin for these proteins. Cloning of the *B. hyodysenteriae nox* gene is an important step towards the goal of generating *B. hyodysenteriae* mutant strains lacking NADH oxidase and for investigating the significance of NADH oxidase in the physiology and pathogenesis of this anaerobic spirochete.

Introduction

Brachyspira (Serpulina) hyodysenteriae is a spirochete and the etiological agent of swine dysentery. Cell products or characteristics hypothesized to be important for *B. hyodysenteriae* to colonize and damage

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mucosal surfaces of the swine cecum and colon include lipooligosaccharide, motility/chemotaxis, hemolysin, attachment, and oxygen utilization [1]. *B. hyodysenteriae* genes encoding hemolytic activity and flagellar proteins have been shown, by insertional inactivation of the genes, to be essential for animal colonization and pathogenesis [2–4].

Brachyspira hyodysenteriae is an aerotolerant anaerobe. Under a $1\%O_2:99\%N_2$ atmosphere, growing cells of the spirochete consume substrate amounts of oxygen [5]. One highly active mechanism (and the only identified) for oxygen uptake in *B. hyodysenteriae* cells is NADH oxidase (Specific activity = 0.8 μmol NADH/min/mg cell protein; [6]). This NADH oxidase is a soluble (not membrane associated), FAD-containing, 47–48-kDa protein that carries out a 4-electron reduction of molecular oxygen to form water [7]. In its biochemical properties, including the N-terminal amino acid sequence, the enzyme resembles the NADH oxidase of another intestinal bacterium, *E. faecalis* [8,9].

The ability to consume oxygen by means of NADH oxidase may enable *B. hyodysenteriae* cells to contend with or take advantage of oxygen in their natural microhabitat, the oxygen-respiring mucosal tissues of the swine intestinal tract. In such a role, the enzyme could be essential for this pathogenic spirochete to colonize and damage intestinal tissues. As a step toward testing this hypothesis, the research described in this article was aimed at cloning and characterizing the *B. hyodysenteriae* NADH oxidase gene (*nox*).

Materials and Methods

Bacterial strains and culture conditions

Escherichia coli DH10B cells (ElectroMAX) were obtained frozen from Gibco-BRL (Gaithersburg, MD, USA). *Brachyspira hyodysenteriae* strains B204 and R-1 originated, respectively, from a dysenteric pig and a diseased rhea in the U.S. and strain A-1 was originally isolated from a dysenteric pig in the U.K. Cells of strains B204, R-1, and A-1 were cultured in BHIS (Brain Heart Infusion Broth containing 10% heat-treated calf serum) medium beneath an initial 1% $O_2:99\%$ N_2 atmosphere [6].

DNA cloning procedures

Initially, portions of the *nox* gene (Figure 1 B–D) were cloned and sequenced and then the entire *nox* gene (Figure 1A) was amplified, sequenced, and cloned. Methods for extracting DNA from *B. hyodysenteriae* cells were modified from those of Marmur [10,11]. Plasmid pCRII, a TA cloning vector, was used to clone both amplified portions of the *nox* gene for preliminary sequencing and eventually the entire gene. Manipulations of the plasmid and conditions for ligation followed recommendations of the manufacturer (TA Cloning Kit, Version 2.2, Invitrogen, San Diego, CA, U.S.A.).

Escherichia coli DH10B cells were transformed with ligated plasmid by electroporation using a Bio-Rad Gene Pulser (2.5 kV; 25 μF)/Pulse Controller (200 ohms) transfection apparatus (Bio-Rad, Richmond, CA, U.S.A.). Cells were electrotransformed in chilled 0.2-cm gap cuvettes following instructions of supplier (Gibco-BRL, Gaithersburg, MD, U.S.A.). Conditions for recovery and selection of recombinant cells by blue-white colony selection followed manufacturer's recommendations. Cells from recombinant colonies were purified by subculturing a single, isolated colony twice, cultured in 10 mL LB broth containing ampicillin (200 μg/mL), and used to make plasmid mini-preps. Cells from 500-mL cultures were harvested by centrifugation, washed, disrupted in a French pressure cell, and assayed for NADH oxidase.

NADH oxidase enzyme assay

Soluble NADH oxidase activities in bacterial cell lysates were assayed spectrophotometrically at least twice by measuring absorbance decreases (340 nm) as NADH was converted to NAD. Methods of analysis and control assays have been described [7,12].

NOX antisera and immunoblotting

Brachyspira hyodysenteriae NADH oxidase was partially purified by ultracentrifugation, $(NH_4)_2SO_4$ precipitation, and dye-ligand affinity chromatography methods as described previously [7]. NADH oxidase was further purified by preparative, nondenaturing, polyacrylamide gel electrophoresis and by monitoring enzyme activity in the electrophoretic gels [7,13]. Sections of the gels containing NADH oxidase activity were cut out, emulsified with Freund's incomplete adjuvant, and used to immunize a germ-free pig [14]. Swine antiserum was designated D40.

Detection of NADH oxidase in *E. coli* cell lysates by immunoblotting (Western blots) followed standard methods [15] as described for detecting *B. hyodysenteriae* whole cell proteins [16]. Membranes containing the blotted cell proteins were incubated with a 1/500 dilution of swine D40 antiserum. Antibodies complexed with the recombinant NADH oxidase were detected by immunoassay using a 1/500 dilution of goat, horseradish peroxidase-conjugated, anti-swine IgG (Bethyl Laboratories, Montgomery, TX, U.S.A.)

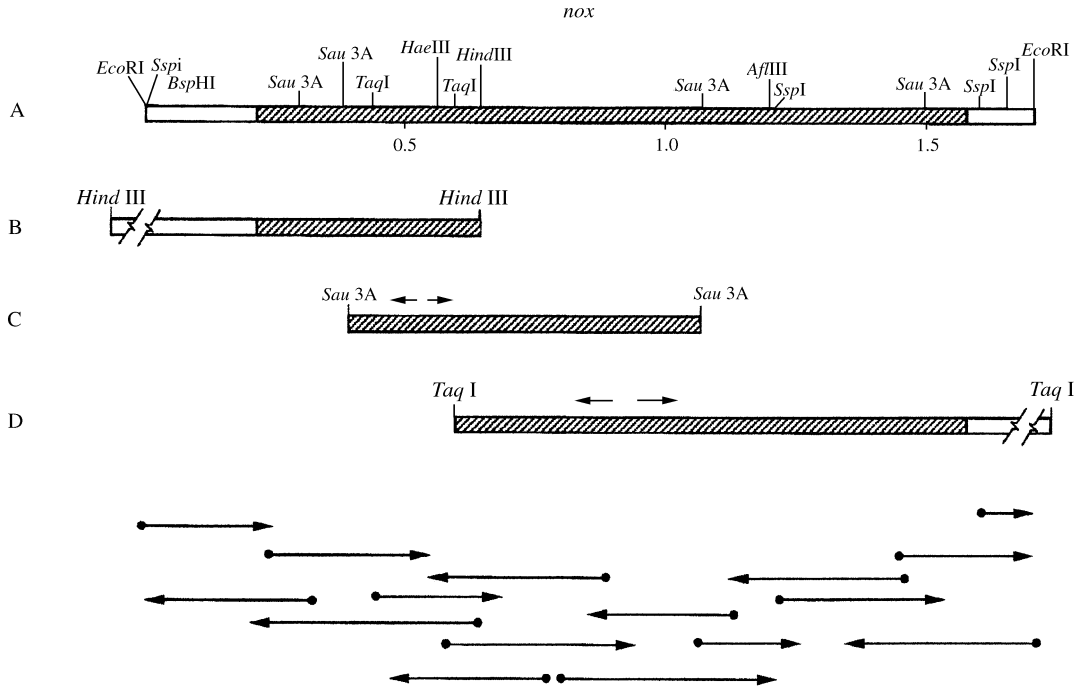

Figure 1. Steps in cloning and sequencing the *B. hyodysenteriae* B204 *nox* gene. (A) Schematic representation of *B. hyodysenteriae nox* gene (hatched areas) and adjacent chromosome regions (open areas) amplified and cloned in plasmid pCRNOX. Numbers indicate length in kb pairs. Sites of selected restriction enzymes are shown. The nucleotide sequence of the DNA is given in Fig. 3. (B) DNA fragment carrying the first 1/3 of the *nox* gene was obtained from a plasmid library of *Hind*III digested DNA from *B. hyodysenteriae*. The clone was detected by using a 20-base oligonucleotide probe hybridizing with the 5′-end of the *nox* gene [12]. (C) Internal portion of the *nox* gene obtained by inverse PCR amplification of *Sau*3A digested *B. hyodysenteriae* DNA. Inverse PCR primer sites are depicted by small arrows. (D) Portion of *nox* gene and adjacent DNA obtained by inverse PCR amplification of *Taq*I-digested *B. hyodysenteriae* DNA. (Bottom) Strategy for PCR cycle sequencing the *nox* gene in portions (B–D) and *in toto*, as a PCR amplification product (A). Primer hybridization sites correspond to the beginings of the large arrows. Regions sequenced in PCR cycle sequencing determinations are depicted by lengths of the large arrows.

and 4-chloro-1-naphthol as the chromogenic substrate.

Sequencing and cloning the NADH oxidase (nox) *gene*

In order to sequence and clone the *nox* gene, portions of the *nox* coding region were cloned directly in the case of a *Hind*III fragment (Figure 1B) or indirectly in the case of *Sau*3A and *Taq*I fragments (Figure 1C, D) which were obtained by amplifying digested *B. hyodysenteriae* DNA by the inverse PCR method [17]. Based on the sequences of cloned fragments, PCR primers were designed for amplifying the *nox* gene. The forward primer, 5′-AAT GCC AAT ATT TTA TAA TAT AA-3′, ended 203 bp upstream of the *nox* start codon ATG and the reverse primer, 5′-TTA TGA TTT TCG TTT TTT AAT T-3′, was complementary to a region starting 98 bp downstream of the *nox* stop codon TAA.

Each PCR amplification mix (100 µl) contained 2.5 mM $MgCl_2$, 100 ng of chromosomal DNA, 2.5 U *Taq* polymerase, 200 µM each of dATP, dTTP, dGTP, and dCTP, 0.2 µM each primer, 10 mM Tris-HCl, pH 8.3, 50 mM KCl, and 0.001% gelatin. A hot start cycle consisting of 2-min template denaturation at 98°C, was followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 48°C for 1 min, and primer extension at 72°C for 2 min. The final cycle had the extension time increased to 10 min to complete synthesis of all strands.

The PCR amplification product (1.7 kb in size) was sequenced, ligated into plasmid pCRII, and cloned into *E. coli*. Plasmid DNA from an *E. coli* strain expressing NADH oxidase activity contained an open reading frame identical in sequence to the *nox* gene sequence predicted from the previously cloned and amplified portions of the gene. The relative positions of primers and the sequencing strategy for the *nox* gene are outlined in Figure 1. Every nucleotide base on either the sense or antisense strand of DNA was sequenced 3–6 times (Figure 1).

All DNA sequences were determined by automated PCR cycle sequencing techniques [18] performed at the Iowa State University Nucleic Acid Facility. The *B. hyodysenteriae* B204 *nox* sequence has been deposited in GenBank under accession number U19610.

Analysis of gene sequences

The *B. hyodysenteriae nox* gene sequence was analysed and compared to database gene sequences by using programs available through the BCM Search Launcher [19], the program Omiga v. 1.1 (Oxford Molecular Group, Inc., Campbell, CA, U.S.A.), and by visual inspection. Through BCM Search Launcher, the programs Clustal W (v1.7), BLASTP (v2.0.4), and BEAUTY post-processer were used to compare, align, and analyze NOX amino acid sequences. Default settings were used for the programs.

Results and Discussion

Cloning and expression of nox *in* E. coli

Following numerous unsuccessful attempts to directly clone the *B. hyodysenteriae nox* gene in plasmid and cosmid vectors, overlapping portions of the gene were cloned and sequenced (Figure 1B–D). The resulting sequence information was used to design PCR primers hybridizing to chromosomal regions upstream and downstream of the gene. After amplification, a PCR product (1.7 kb) representing the *nox* gene and several hundred bp of flanking chromosomal DNA (Figure 1A) was purified and sequenced. The product was also inserted into plasmid pCRII and cloned into *E. coli* DH10B cells by electroporation. Of 40 potential recombinant colonies, two contained plasmids that were 5.6 kb (3.9 kb pCRII + 1.7 kb insert). The first tested of these, strain NX3, had detectable NADH oxidase activity and was selected for subsequent analyses.

The plasmid in strain NX3 was designated pCRNOX. Analysis of restriction fragment patterns of purified pCRNOX after digestion by the enzymes *Eco*RI, *Hind*III, *Afl*III, and *Bsp*HI, indicated that pCRNOX contained a 1.7 kb DNA insert DNA with restriction enzyme sites consistent with those expected for the *B. hyodysenteriae nox* gene and flanking chromosomal DNA (Figure 1A). Restriction fragment analysis also indicated the cloned PCR product had been inserted into vector pCRII in a reverse orientation, that is, with the sense strand of the *nox* gene ligated to the antisense strand of the *lacZα* gene and the first codon of the *nox* ORF located approximately 180 bases from the T7 promoter region in pCRNOX (not shown).

Expression of B. hyodysenteriae *NADH oxidase in* E. coli

Cell lysates of *E. coli* strain NX3 contained a protein with a molecular mass of approximately 48 kDa that was absent from cell lysates of control strain B-1 derived by transforming *E. coli* DH10B with plasmid pCRII (Figure 2A). As determined by Western immunoblots (Figure 2B), this protein reacted with swine antiserum raised against purified *B. hyodysenteriae* NADH oxidase. NADH oxidase purified from *B. hyodysenteriae* migrates as a 47–48 kDa protein during SDS-PAGE [7].

Escherichia coli NX3 cell lysates had soluble NADH oxidase activity (Table 1). As with *B. hyodysenteriae* cell lysates, the activity increased after ultracentrifugation, indicating much of the activity was soluble (not membrane associated). In contrast to these results, cell lysates of the control strain *E. coli* B-1 expressed NADH oxidase activity that was removed by ultracentrifugation and thus likely represents endogenous, membrane-associated, cytochrome-linked NADH dehydrogenase activity (Table 1). NADH oxidase activity in *E. coli* NX3 cell supernates was not detected under anaerobic assay conditions (data not shown), consistent with oxygen being an essential electron acceptor for NADH oxidation.

Brachyspira hyodysenteriae *NADH oxidase (*nox*) gene sequence*

The *nox* sequence (Figure 3) was compiled from sequences of portions of the gene (Figure 1B–D), from

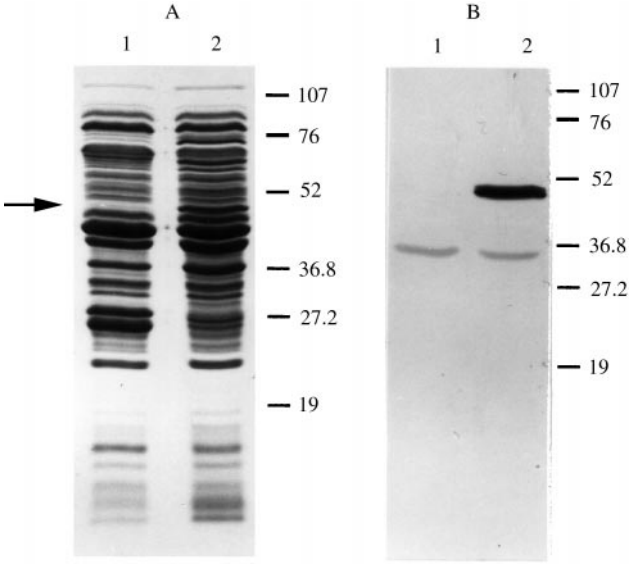

Figure 2. Expression of *B. hyodysenteriae* NADH oxidase protein in *E. coli*. (A) Proteins in *E. coli* cell lysates were separated by SDS-PAGE and stained with Coomassie blue. Each lane contained 10–12 μg of total cell protein. Lane 1: *E. coli* strain B-1 (control strain containing plasmid pCRII). Lane 2: *E. coli* strain NX3 (containing plasmid pCRNOX). Protein equal to *B. hyodysenteriae* NOX in molecular mass (approximately 50 kDa) in NX3 cell lysates is indicated by arrow. (B) Western immunoblot of proteins from gel in A. Membrane was incubated with swine antiserum reacting with purified *B. hyodysenteriae* NADH oxidase and then incubated with goat anti-swine IgG labelled with horseradish peroxidase. Blots were developed with 4-chloro-1-naphthol.

Table 1. NADH oxidase activity in cell lysates of *B. hyodysenteriae* and *E. coli* strains

Bacterial strain	Plasmid content	NADH oxidase activity[a]	
		Cell lysate (before ultracentrifugation)	Cell supernate (after ultracentrifugation)
B. hyodysenteriae B204	none	1.4	3.3
E. coli B-1 (control)	pCRII	0.4	ND (<0.05)
E. coli NX3	pCRNOX	1.5	2.0

[a]NADH oxidase activity in the table is expressed as μmoles NADH oxidized/min/mg cell protein. Soluble oxidase activity (not membrane-associated) remains in the supernate after cell lysates are ultracentrifuged at 140 000 × *g* for 2 h. ND = not detected.

the sequence of the 1.7 kb PCR product, and from the sequence of the pCRNOX plasmid insert. The G+C content of *nox* is low (33.7 %), as expected for a gene of *B. hyodysenteriae*, an organism with a DNA G+C content of 26 mol %.

The *nox* genes of two additional *B. hyodysenteriae* strains, A-1 and R-1, were amplified and the amplification products sequenced. The coding sequence of the *nox* gene from strain A-1 was identical to that of strain B204. The R-1 *nox* gene differed at one nucleotide position (T for C at base position 727 in Figure 3) from that of the other two strains. However, the corresponding amino acid of the R-1 NOX protein at that position, due to codon degeneracy, would not be affected. Thus, the *nox* gene sequences of three *B. hyodysenteriae* strains originating from different animal hosts (swine, rhea) and different geographical locations (U.S.A., U.K.) are highly conserved. For this reason, the *nox* gene might be a good target for designing gene probes or PCR reactions to be used in the clinical identification of *B. hyodysenteriae*, if the *nox* genes of other *Serpulina* species are sufficiently different in sequence.

Brachyspira hyodysenteriae *NADH oxidase predicted amino acid sequence*

The 17 amino acids at the N-terminal end of the NOX protein, as predicted from the translated DNA sequence (Figure 3), are identical to the terminal amino acids of the purified NADH oxidase [7]. The translated amino acid sequence is predicted to encode a protein with a relative molecular mass of 50 168, close to the 47–48 kDa estimated for the purified NADH oxidase protein [7] .

Based on an analysis of GenBank sequences, the *B. hyodysenteriae* NOX protein shares significant amino acid sequence identity, 46% and 47%, respectively, with NADH oxidases from *E. faecalis* and *S. mutans*, consistent with common properties of the enzymes [8,9,20–22]. Several regions of the *B. hyodysenteriae*, *E. faecalis*, and *S. mutans* NADH oxidases displayed greater than 50% amino acid sequence identity (Figure 4, A–G), an indication of protein domains essential for enzyme activity. Two regions of the *E. faecalis* protein (Figure 4, A & X), are likely to be involved in binding of FAD [9,20]. The *E. faecalis* NADH oxidase contains cys42 (Figure 4, Region B) which serves as a redox center of the enzyme, participating with FAD in the reduction of oxygen to water [23–25]. His10 (Figure 4, Region A) is conserved in comparable NADH oxidases and interacts with transition state peroxide in the oxidase reaction [25]. Region D contains a glycine-rich GXGXXG sequence, typical of an ADP-binding domain associated with binding of NADH [9,26]. Other conserved regions may be important for maintaining the structural integrity of the enzymes. Based on their common functional domains and overall sequence similarities, the *nox* genes of the spirochete *B. hyodysenteriae* and of these Gram-positive cocci seem likely to share a common ancestral origin.

Widespread distribution of nox *genes among micro-organisms*

NADH oxidase, the *nox* gene, or both have been identified in various bacterial species (listed in [12]); in the mitochondria-lacking, parasitic protozoan, *Giardia duodenalis* [27]; and in 43 strains of intestinal spirochetes [12], now known to represent six species of *Serpulina*. Putative *nox* genes are present in the genome sequences of *Mycoplasma pneumoniae* [28], *M. genitalium* [29], *Borrelia burgdorferi* [30], and *Treponema pallidum* [31].

NADH oxidase activities have not yet been directly demonstrated for proteins encoded by the putative *nox* genes of *T. pallidum* and *B. burgdorferi*. Nevertheless, this enzyme is the only mechanism of oxygen metabolism recognized during genome sequencing of these human pathogenic spirochetes [30,31]. The enzyme is a major, and perhaps the only, oxygen consuming activity in *B. hyodysenteriae* cells [6]. A reasonable hypothesis is that NADH oxidase contributes to the ability of these anaerobic/microaerophilic

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   1    A ATG CCA ATA TTT TAT AAT ATA AAC ATT TTT TGT AAA ATT TAT ATA CAT TTA ATG CTT      58

  59  TTA ATT ATC ATA ATT CAT GAT AAT TAG TGA AAT CAT TAT ATA AAA AAC ATA CTA AAA CTA     118

 119  TTA AAA TTT ACT AAG TTA CAT ATA ATA TAA CTT GAC TAA GTA TTT TTT TTG TAC TAT AAT     178

   1                                                                Met Lys Val Ile       4
 179  AAA CAC CAA TTT TAT ATT AGA TTA TTT TTA ATA AGG GGT TAA ATT ATT ATG AAA GTT ATT     238

   5  Val Ile Gly Cys Asn His Ala Gly Thr Trp Ala Ala Lys Thr Leu Lys Ala Thr Asp Pro      24
 239  GTA ATA GGT TGT AAC CAT GCT GGT ACA TGG GCA GCA AAA ACT TTG AAA GCT ACA GAT CCT     298

  25  Asn Cys Gln Val Val Thr Tyr Asp Arg Asn Asp Asn Ile Ser Phe Leu Ala Cys Gly Ile      44
 299  AAT TGT CAA GTA GTT ACT TAC GAT AGA AAT GAT AAT ATA TCT TTC TTA GCC TGC GGT ATC     358

  45  Ala Leu Trp Val Gly Gly Val Val Lys Asp Pro Lys Gly Leu Phe Tyr Ala Ser Pro Glu      64
 359  GCA CTT TGG GTT GGT GGC GTA GTT AAA GAT CCT AAA GGA TTA TTC TAT GCT AGT CCT GAA     418

  65  Ser Leu Arg Gly Glu Gly Ile Asp Val Tyr Met Gly His Asp Val Thr Lys Ile Asp Trp      84
 419  AGT TTG AGA GGT GAA GGC ATC GAT GTT TAT ATG GGA CAT GAT GTT ACT AAA ATA GAC TGG     478

  85  Ala Asn Lys Lys Leu Cys Val Lys Glu Leu Lys Thr Gly Lys Glu Phe Glu Asp Thr Tyr     104
 479  GCT AAC AAA AAA TTA TGT GTA AAA GAA CTA AAA ACA GGA AAA GAG TTT GAA GAC ACT TAC     538

 105  Asp Lys Leu Ile Leu Ala Thr Gly Ser Trp Pro Val Thr Pro Pro Ile Glu Gly Leu Lys     124
 539  GAT AAA CTT ATT CTT GCT ACT GGT TCT TGG CCT GTA ACT CCT CCT ATC GAA GGC TTA AAA     598

 125  Gln Glu Gly Thr Thr Tyr Gly Leu Lys Lys Gly Ile Phe Phe Ser Lys Leu Tyr Gln Gln     144
 599  CAA GAA GGA ACT ACT TAC GGA CTT AAA AAA GGT ATT TTC TTC TCT AAG CTT TAT CAG CAA     658

 145  Gly Gln Glu Ile Ile Asp Glu Ile Ala Lys Pro Asp Val Lys Lys Val Met Val Val Gly     164
 659  GGA CAA GAA ATT ATT GAT GAA ATA GCT AAA CCA GAT GTT AAA AAA GTT ATG GTA GTT GGT     718

 165  Ala Gly Tyr Ile Gly Val Glu Leu Ile Glu Ala Phe Lys Asn His Gly Lys Glu Val Ile     184
 719  GCT GGA TAC ATA GGT GTT GAA CTT ATA GAA GCA TTC AAA AAC CAT GGT AAA GAA GTT ATC     778

 185  Leu Met Glu Ala Met Pro Arg Val Met Ala Asn Tyr Phe Asp Lys Glu Ile Thr Asp Glu     204
 779  TTA ATG GAA GCT ATG CCT AGA GTT ATG GCT AAC TAC TTT GAT AAA GAA ATC ACT GAT GAA     838

 205  Ala Glu Lys Arg Ile Lys Glu Ala Gly Ile Glu Met His Leu Gly Glu Thr Val Lys Lys     224
 839  GCT GAA AAA AGA ATC AAA GAA GCT GGC ATA GAA ATG CAT TTA GGT GAA ACT GTT AAG AAA     898

 225  Phe Glu Gly Asp Asp Arg Val Lys Lys Val Val Thr Asp Lys Gly Ser Tyr Asp Val Asp     244
 899  TTT GAA GGT GAT GAC AGA GTT AAA AAA GTT GTT ACT GAC AAA GGT TCT TAT GAT GTA GAT     958

 245  Met Val Val Met Ser Val Gly Phe Arg Pro Asn Asn Glu Leu Tyr Lys Asp Tyr Leu Glu     264
 959  ATG GTA GTT ATG TCT GTT GGT TTC AGA CCT AAT AAT GAA CTT TAT AAA GAT TAT TTA GAA    1018

 265  Thr Leu Pro Asn Gly Ala Ile Val Val Asp Thr Thr Met Lys Thr Thr Lys Asp Pro Asp     284
1019  ACT TTA CCT AAT GGT GCT ATT GTA GTA GAT ACT ACT ATG AAA ACT ACT AAA GAT CCT GAT    1078

 285  Val Phe Ala Ile Gly Asp Cys Ala Thr Val Tyr Ser Arg Ala Ser Glu Lys Gln Glu Tyr     304
1079  GTA TTT GCT ATA GGT GAC TGT GCT ACT GTA TAT TCA AGA GCT TCT GAA AAA CAA GAA TAT    1138

 305  Ile Ala Leu Ala Thr Asn Ala Val Arg Met Gly Ile Val Ala Ala Asn Asn Ala Leu Gly     324
1139  ATT GCT TTA GCT ACT AAT GCT GTA AGA ATG GGT ATT GTT GCT GCT AAT AAT GCT TTA GGA    1198

 325  Lys His Val Glu Tyr Cys Gly Thr Gln Gly Ser Asn Ala Ile Cys Val Phe Gly Tyr Asn     344
1199  AAA CAT GTT GAA TAT TGC GGT ACT CAA GGT TCT AAT GCT ATT TGT GTA TTT GGA TAC AAT    1258

 345  Met Ala Ser Thr Gly Trp Ser Glu Glu Thr Ala Lys Lys Lys Gly Leu Lys Val Lys Ser     364
1259  ATG GCT TCT ACT GGT TGG TCT GAA GAA ACT GCT AAG AAA AAA GGA TTA AAA GTA AAA TCT    1318

 365  Asn Phe Phe Lys Asp Ser Glu Arg Pro Glu Phe Met Pro Thr Asn Glu Asp Val Leu Val     384
1319  AAC TTC TTC AAA GAT TCT GAA AGA CCA GAA TTT ATG CCT ACT AAT GAA GAT GTT TTA GTA    1378

 385  Lys Ile Ile Tyr Glu Glu Gly Ser Arg Arg Leu Leu Gly Ala Gln Ile Ala Ser Lys His     404
1379  AAA ATC ATT TAT GAA GAA GGC AGC AGA CGT TTA TTA GGT GCT CAA ATA GCT TCT AAA CAC    1438

 405  Asn His Ala Glu Ala Ile His Ala Phe Ser Leu Ala Ile Gln Asn Gly Met Thr Val Asp     424
1439  AAT CAT GCT GAA GCT ATT CAT GCA TTC TCT CTT GCT ATA CAA AAT GGT ATG ACT GTT GAT    1498

 425  Gln Phe Ala Leu Ser Asp Phe Phe Phe Leu Pro His Tyr Asn Lys Pro Leu Ser Trp Met     444
1499  CAA TTT GCA TTG TCA GAT TTC TTC TTC CTA CCT CAC TAC AAC AAA CCA TTA TCT TGG ATG    1558

 445  Thr Met Val Ala Tyr Thr Ala Lys ***                                               452
1559  ACT ATG GTT GCT TAT ACT GCT AAA TAA TTA TAG AAA ATA TAA TTA GTT TAA GAA TAT TTA    1618

1619  CCC CCT AAT AAA TTT ATT TTA TTA GGG GGT TTT TAT AAT CAA TAA ATA TTT TAC TCA CAT    1678

1679  ATA TAA ATT AAA AAA CGA AAA TCA TAA                                               1705
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Figure 3. Nucleotide sequence and translated amino acid sequence of the *nox* gene of *B. hyodysenteriae*. Computer-predicted transcription start and ribosome binding sites for *E. coli* are indicated by bold, large letter "A" and double underline, respectively. Shaded sequences represent inverted repeat sequence likely to be associated with transcription termination ($\Delta G = -25.5$ kCal/mol).

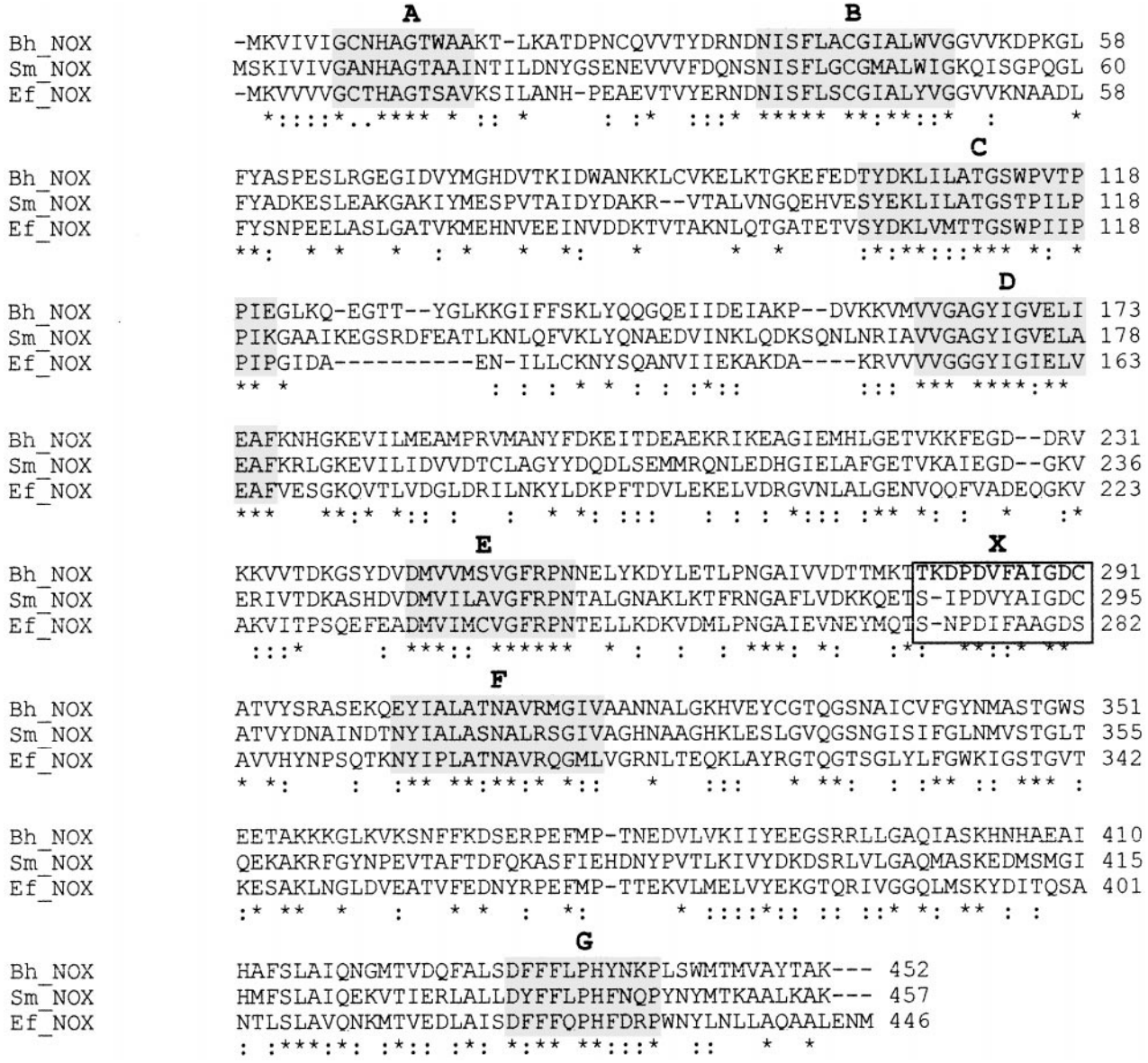

Figure 4. Sequence comparisons of NADH oxidases from *B. hyodysenteriae* (Bh; Genbank U19610), *Enterococcus faecalis* (Ef; Genbank S45681), and *Streptococcus mutans* (Sm; D49951). The symbol (*) indicates a position with three identical amino acids and (:) indicates a position with either two identical and one similar or three similar amino acids. Shaded regions A-G have greater than 50% identity over at least 10 amino acids and no sequence gaps. The A and X regions of *E. faecalis* NADH oxidase are involved in FAD binding [9,20].

spirochetes to persist among the oxygen-respiring tissues of their animal hosts.

Conclusions

Although NADH oxidase is commonly present in various species of host-colonizing anaerobic and micro-aerophilic bacteria, the significance of this enzyme in the lifestyles of these host-colonizing microbes has not been established. The cloning and characterization of the *nox* gene of *B. hyodysenteriae*, as reported in this article, will likely facilitate studies of NADH oxidase expression by *B. hyodysenteriae* cells, the generation of *B. hyodysenteriae* mutant strains deficient in oxidase activity, and an evaluation of the ability of the mutant strains to colonize and damage intestinal tissues. These are essential steps in identifying selective advantages and determining the specific role of NADH oxidase for *B. hyodysenteriae* in its natural environment, the swine intestinal tract.

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